A Simplified Electrometric Technique For Rapid Measurement Of Human Blood Cholinesterase Activity

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Abstract

A simplified electrometric method was described and validated for measurement of human plasma and erythrocyte cholinesterase (ChE) activities. A suitable ChE assay mixture in a 10-ml beaker contained 0.2 ml of plasma or erythrocytes, 3 ml of distilled water, 3 ml pH 8.1 barbital-phosphate buffer and 0.1 ml of 7.5% acetylthiocholine iodide as a substrate. The one-step incubation was for 20 minutes at 37 °C. Replicate assay for the precision of the electrometric method produced a coefficient of variation 5.5% in the plasma and 6.5% in the erythrocytes. Reference ChE activities (pH/30 min) in the plasma and erythrocytes of male (1.05 and 1.18) and female (0.91 and 1.19) subjects were also obtained. There were no significant differences in plasma and erythrocyte ChE activities when heparin and disodium EDTA were used as anticoagulants. The estimated percentage of true ChE activity in the plasma after inhibition of pseudo ChE by quinidine sulfate was 21.4%. Six subjects with routine exposure to insecticides because of their work had 38 and 26% decreases in plasma and erythrocyte ChE activities, respectively. The organophosphate insecticide dichlorvos (0.125-4 µM) and the carbamate insecticide carbaryl (5-20 µM) significantly inhibited plasma and erythrocyte ChE activities in vitro. Oral administration of dichlorvos (25 and 50 mg/kg) and carbaryl (100 and 200 mg/kg) in mice induced signs of toxicosis characteristic of cholinergic overstimulation, and these signs were associated with significant decreases in plasma, erythrocyte and brain ChE activities as measured by the present electrometric method.. The results suggested that the present electrometric method for measurement of blood ChE activity in man is simple, accurate, and efficient in monitoring the enzyme inhibition caused by organophosphate and carbamate insecticides.

INTRODUCTION

Organophosphate and carbamate insecticides are widely used in veterinary practice and in agriculture ([1,2,3,4]). They pose major environmental pollution problems and health hazards to man and animals ([2,3,4,5]). The most important toxic action of organophosphate and carbamate insecticides is inhibition of acetylcholinesterase activity leading to accumulation of acetylcholine at the nerve endings and subsequently causing cholinergic overstimulation characterized by nicotinic, muscarinic and central nervous system effects ([1,2,3,6,7]).

Measurement of blood or tissue cholinesterase activity is an ideal biomarker for monitoring exposure to organophosphate and carbamate insecticides and for diagnosing poisoning induced by these pesticides ([4,8,9,10,11,12,13]). Usually a 20-30% decrease in blood ChE activity indicates exposure to ChE inhibitors ([2,7,13]), and further enzyme inhibition is an indication of hazardous condition and poisoning ([2,6,7]).

Various colorimetric and electrometric methods exist for measurement of ChE activity in the blood and other tissues such as the brain ([8,9,10,14]). One of the common methods for measuring blood ChE activity is the electrometric method of Michel which is based on the hydrolysis of acetylcholine and production of acetic acid which in turn decreases the pH of the reaction medium ([15]). However, the shortcomings of the Michel's electrometric method are relative insensitivity, sample size, long incubation period and low throughput ([8,9,10]). In addition, the original electrometric method is not preferred for detection of ChE inhibition induced by carbamates ([9,16,17]). Carbamylated ChE is unstable in the reaction mixture of the electrometric method of Michel because of considerable sample dilution and long pre-reaction and post-reaction incubation times (totally >60 minutes) ([16,17,18]).

Different modifications of the electrometric method have been described for measuring plasma or erythrocyte ChE activities in clinical and research applications ([8,9,10,14,19]). These modifications include increasing sample volume,

increasing or decreasing incubation time, increasing incubation temperature or using buffers of different compositions (for review see [8,9,10,11,14]). A simple modification of the electrometric method is that of Mohammad et al. ([19]) which was advocated for its one-step short incubation time (30 minutes), the use of a single barbital-phosphate buffer and the overall shorter assay time by omitting apparently unnecessary steps of saline washing of the erythrocytes and the one or two incubation periods of 10 minutes each before substrate addition. The method has been applied efficiently on several animal species such as mice ([20]), rats ([21]), rabbits ([22]), goats ([23]), chickens ([24,25]), and wild birds ([26]). Further, the present method, in contrast to the original electrometric method of Michel ([15]), can detect ChE inhibition caused by carbamate insecticides such as carbaryl ([24,25]) and methomyl ([21]). The method correlates well with the electrometric method of Michel ([19]) and the spectrophotometric method of Ellman ([24]) in measuring ChE activity. No attempts have been made to use the present method for measuring ChE activity in man. Therefore, the purpose of the present study was to validate and apply the method of Mohammad et al ([19]) in measuring blood ChE activity in humans.

SUBJECTS AND METHODS

SUBJECTS

Human volunteers included in the study were apparently healthy subjects with no history of exposure to antiChE insecticides. Six subjects with a history of exposure to insecticides during their routine work were also included in the study. They were veterinarians and agriculture workers. However, the frequency of exposure to insecticides and /or other chemicals could not be verified from these latter volunteers. Informed consents were obtained from the volunteers before the start of the study.

MICE

Male albino Swiss mice weighing 20-35 g were housed under standard laboratory conditions at a temperature of 22±2 °C and 10/14 hours light-dark cycle with water and food available ad libitum.

Electrometric method for measurement of plasma or erythrocyte ChE activity:

Venous blood samples were collected from human volunteers using heparinized test tubes ([27]). Plasma was separated from erythrocytes by centrifugation at 3000 rpm (Centurion, U.K.) for 15 minutes. For a typical assay condition, the reaction mixture in a 10-ml beaker contained 3 ml distilled water, 0.2 ml plasma or erythrocytes and 3 ml, pH 8.1 barbital-phosphate buffer ([19]). the ph of the mixture (ph1) was measured with a glass electrode using a ph meter (consort, belgium), then 0.1 ml of aqueous solution of acetylthiocholine iodide (7.5%) was added to the reaction mixture which was incubated at 37 °c for 20 minutes. at the end of the incubation period, the ph of the reaction mixture (ph2) was measured. the enzyme activity was calculated as follows:

ChE activity (pH/20 minutes) = (pH1 – pH2) - pH of blank

The blank was without plasma or erythrocytes. The pH 8.1 buffer consisted of 1.237 g sodium barbital (BDH), 0.63 g potassium dihydrogen phosphate (E-Merck. Dermstadt, Germany) and 35.07 g sodium chloride (BDH)/1 L of distilled water ([19]).

For measurement of brain ChE activity in mice, the tissue sample was homogenized in the pH 8.1. barbital-phosphate buffer at 3 ml/100 mg wet weight ([20, 24]) with a teflon homogenizer (Karl Kolb, Germany) using 25% of the maximum velocity of the homogenizer. Homogenization was performed on an ice bath, and brain homogenates were kept on ice before ChE determination. For brain ChE activity, 0.2 ml of the tissue homogenate was used instead of the plasma aliquot in the reaction mixture described above.

Preliminary experiments using pooled human plasma or erythrocyte samples indicated that an incubation time of 20 min after the addition of the substrate (either 7.5% aqueous solution of acetylthiocholine iodide or 7.1 % aqueous solution of acetylcholine iodide) and a sample volume of 0.2 ml were suitable for measuring the ChE activity at 37 °C. The experiments described below were performed to standardize the present electrometric method in man, and to demonstrate its precision, reproducibility, validity and other specifications. The described method was applied in mice to measure ChE inhibition induced by two commonly used insecticides, dichlorvos, an organophosphate and carbaryl, a carbamate.

PRECISION OF THE DESCRIBED ELECTROMETRIC METHOD

Plasma and erythrocyte samples obtained from 9 subjects of either sex were pooled separately. Plasma and erythrocyte ChE activities were determined for 10 times. The mean, standard deviation, standard error, coefficient of variation and 95% confidence interval were calculated ([28]).

NORMAL REFERENCE RANGE VALUES OF PLASMA AND ERYTHROCYTE CHE ACTIVITIES

Blood samples were obtained from both male and female subjects (10-55 years old) to measure ChE activity in the plasma and erythrocytes. ChE activities in the samples were measured by the described method to determine for the first time preliminary reference range values.

ANTICOAGULANTS

Blood samples were collected from 7 subjects of either sex; each sample was collected in two test tubes. One tube contained disodium EDTA (1 mg/ ml blood) and the other one contained heparin as anticoagulants ([27]). Plasma and erythrocyte aliquots obtained from these samples were used for ChE determination.

TRUE CHE ACTIVITY IN THE PLASMA

Plasma samples of 9 subjects of either sex were individually divided into two portions. The first portion was used for measuring the ChE activity as described before. To the reaction mixture of the second portion, 40 µl of 0.1% quinidine sulfate (Sigma, St. Louis, USA) was added, and incubated for 10 minutes at 37 °C ([19,24]). Quinidine specifically inhibits pseudo ChE activity in the plasma ([10,11,12]). Following the 10-min incubation period for inhibiting pseudo ChE activity ([19,24]), the remaining activity (true ChE) was measured as before. Pseudo ChE activity= ChE activity (without quinidine) – true ChE activity (with quinidine).

IN VITRO CHE INHIBITION BY ORGANOPHOSPHATE (DICHLORVOS) AND CARBAMATE (CARBARYL) INSECTICIDES

Plasma or erythrocyte samples were collected from 4-6 volunteers of either sex. The method of inhibitor-ChE incubation ([19,24]) was used to measure the in vitro inhibition of plasma and erythrocyte ChE activities by dichlorvos (50% EC, Al-Tariq Co., Iraq) and carbaryl (85% powder, Sociedad Anonima De Agroquimicos, Spain). The insecticides were prepared in distilled water and individually added in a volume of 0.1 ml to the reaction mixtures of the plasma or erythrocytes. The final amounts of dichlorvos in the reaction mixtures were 0 (base-line control), 0.125, 0.25, 0.5, 1, 2 and 4 µM, whereas those of carbaryl were 0 (base-line control), 5, 10 and 20 µM. The reaction mixtures containing the insecticides were incubated at 37 °C for 10 minutes. Thereafter, the residual ChE activity in the mixture was measured as before. The % of enzyme inhibition was calculated as follows:

$$\% \text{ ChE inhibition} = \frac{\text{ChE activity (without insecticide)} - \text{ChE activity (with insecticide)}}{\text{ChE activity (without insecticide)}} \times 100$$

CHE ACTIVITY OF SUBJECTS EXPOSED TO INSECTICIDES

Blood samples were collected from 6 male subjects who were exposed to antiChE insecticides during their routine work as veterinarians and agriculture workers. However, none of the subjects had overt signs of illnesses. Their blood ChE activities were statistically compared with the reference range values described above.

CHE INHIBITION BY DICHLORVOS AND CARBARYL IN MICE

Mice were treated orally by a gavage needle with either dichlorvos (5/group) at the dose rates of 0 (distilled water-control), 25 and 50 mg/kg body weight or with carbaryl (6/group) at the dose rates of 0 (distilled water-control), 100 and 200 mg/kg body weight. Distilled water was used to freshly prepare the required concentrations of the insecticides and the volume of administration was at 10 ml/kg. The doses of the insecticides were obtained from literature ([3,29]) as well as from preliminary experiments conducted in mice. After dosing, the mice were monitored for the appearance of signs of poisoning characteristics of cholinergic poisoning ([21,30]). Two hours after the dosing, the mice were anesthetized with ether to obtain blood and then killed by cervical dislocation to excise the brain for ChE measurement as described above.

STATISTICS

When applicable the data were subjected to analysis of variance followed by the least significant difference test ([30]). Student's-t-test was used for the means of two groups. The level of significance was at $P < 0.05$. Other statistical calculations used in the present study are found elsewhere ([28]).

RESULTS

The coefficient of variation of the described electrometric method in measuring plasma and erythrocyte ChE activities were 5.5% and 6.5%, respectively (Table 1).

Figure 1

Table 1: Precision of the described electrometric method for measurement of plasma and erythrocyte cholinesterase activities (pH/20 minutes)

Parameter	Plasma	Erythrocyte
No. of replicates	10	10
Mean	1.02	1.31
Standard deviation	0.056	0.085
Standard error	0.018	0.027
Coefficient of variation	5.5%	6.5%
95% confidence interval	0.99, 1.06	1.26, 1.36

REFERENCE RANGE OF CHE ACTIVITY

Table 2 shows the mean, 95% confidence interval and related statistics for plasma and erythrocyte ChE activities of apparently healthy males and female subjects. The mean plasma ChE activities (pH/20 min) of the males and females were 1.05 and 0.91, respectively, whereas those of the erythrocytes were 1.18 and 1.19, respectively. There were no significant differences between male and female ChE values.

Figure 2

Table 2: Reference cholinesterase activities (pH/20 minutes) in the plasma and erythrocytes of male and female subjects

Parameter	Male		Female	
	Plasma	Erythrocyte	Plasma	Erythrocyte
N	40	45	41	41
Mean	1.05	1.18	0.91	1.19
Standard deviation	0.153	0.127	0.185	0.149
Standard error	0.024	0.019	0.029	0.023
95% confidence interval	1.00, 1.10	1.14, 1.22	0.85, 0.97	1.14, 1.24
Range	1.37-0.77= 0.60	1.53-0.94= 0.59	1.31-0.59= 0.72	1.48-0.95= 0.53

ANTICOAGULANTS

There were no significant differences in the mean±SE of plasma (0.94±0.07 and 0.89±0.06 pH/20 min) and erythrocyte (1.31±0.04 and 1.31±0.05 pH/20 min) ChE activities when heparin and EDTA were used as anticoagulants, respectively.

TRUE PLASMA CHE ACTIVITY

Using quinidine sulfate to inhibit pseudo ChE activity in the plasma, the percentage of true ChE activity was estimated to be 21.4% (Table 3).

Figure 3

Table 3: Estimation of true cholinesterase activity (pH/20 minutes) in the human Plasma

Variable	Mean ± SE	% Activity
Total cholinesterase	0.84 ± 0.086	100
True cholinesterase*	0.18 ± 0.019	21.4
Pseudo cholinesterase	0.66 ± 0.079	78.6

N= 9 plasma samples.
* Quinidine sulfate was used to inhibit pseudo cholinesterase activity in the plasma.

CHE ACTIVITY OF SUBJECTS EXPOSED TO ANTICHE INSECTICIDES

Mean (± SE) of plasma and erythrocyte ChE activities of subjects exposed to antiChE insecticides during their routine work were 0.65±0.07 and 0.87±0.09, respectively. These values were below respective reference values by 38 and 26%, respectively. However, the subjects did not complain from any overt adverse effects.

IN VITRO CHE INHIBITION

The insecticides dichlorvos and carbaryl significantly and in a concentration-dependent manner inhibited human plasma and erythrocyte ChE activities in vitro (Table 4). The percentage of plasma ChE inhibition in case of dichlorvos was more than that of the erythrocyte within each inhibitor concentration (Table 4).

Figure 4

Table 4: In vitro inhibition of human plasma and erythrocyte cholinesterases (ChE) by dichlorvos and carbaryl

Inhibitor concentration (µM)	Plasma ChE		Erythrocyte ChE	
	ΔpH/20 minutes	% inhibition	ΔpH/20 minutes	% inhibition
Dichlorvos				
0	1.01± 0.026	0	1.15± 0.055	0
0.125	0.17± 0.032*	83	0.89± 0.056*	23
0.25	0.11± 0.017*	89	0.73± 0.059*	37
0.50	0.10± 0.005*	90	0.55± 0.034*	52
1.0	0.08± 0.009*	92	0.43± 0.047*	63
2.0	0.07± 0.007*	93	0.32± 0.047*	72
4.0	0.05± 0.011*	95	0.21± 0.033*	82
Carbaryl				
0	1.12±0.120	0	1.21±0.020	0
5	0.70±0.080*	38	0.72±0.040*	41
10	0.57±0.077*	49	0.60±0.045*	50
20	0.39±0.070*	65	0.46±0.040*	62

N = 4 / concentration group.
ChE values are mean±SE.
* Significantly different from the respective control (0 concentration), P< 0.05.

CHE INHIBITION BY DICHLORVOS AND CARBARYL IN MICE

Oral dosing of mice with dichlorvos at 25 and 50 mg/kg induced signs of cholinergic poisoning manifested as excitation, straub tail, gasping, lacrimation, salivation, flat body appearance as well as tremors and muscle fasciculation as well as convulsion at the high dose. Dichlorvos treatments significantly inhibited plasma ChE by 46 and 47%, respectively in comparison with control values (Table 5). Erythrocyte and brain ChE activities also decreased, though non-significantly, by 30 and 33%, respectively when compared with control values (Table 5).

Figure 5

Table 5: Cholinesterase (ChE) inhibition in mice dosed orally with dichlorvos and Carbaryl

Dose (mg/kg)	Plasma ChE		Erythrocyte ChE		Brain ChE	
	ΔpH/20 minutes	% inhibition	ΔpH/20 minutes	% inhibition	ΔpH/20 minutes	% inhibition
Dichlorvos						
0	1.13±0.01	0	0.33±0.03	0	0.21±0.02	0
25	0.61±0.05*	46	0.23±0.04	30	0.14±0.03	33
50	0.60±0.03*	47	0.23±0.04	30	0.14±0.03	33
Carbaryl						
0	0.92±0.16	0	0.34±0.05	0	0.21±0.02	0
100	0.67±0.10	27	0.26±0.01	24	0.14±0.02*	33
200	0.51±0.08*	45	0.19±0.02*	44	0.14±0.02*	33

ChE values are mean±SE of 5 mice/group in case of dichlorvos and 6 mice/group in case of carbaryl.
* Significantly different from the respective control value, P< 0.05.

Oral dosing of mice with carbaryl at 100 and 200 mg/kg also induced signs of cholinergic poisoning and dose-dependently inhibited plasma (27 and 45%) and erythrocyte (24 and 44%) ChE activities in comparison with respective control values (Table 5). Both carbaryl treatments significantly inhibited brain ChE by 33% in comparison with the control value (Table 5).

DISCUSSION

Measurement of blood ChE activity in man and animals is a non-invasive biomarker method for monitoring poisoning or exposure to organophosphate and carbamate insecticides ([4,8,9,10,11,12,13]). The ChE methods usually offer specificity and simplicity ([8,9,10,11,12,13,14]). However, methods available for measuring ChE activity suffer from a wide range of variability and difficulties in reproducibility ([8,9,10]). Further, the shortcomings of the original Michel's electrometric method are relative insensitivity, sample size and low throughput ([8,9,10]). What complicates the matter is that the Michel's method is not directly applicable to measure animal ChE which differs considerably from that of the human ([9,10,11,14,17]). Therefore, several investigators advocated many modifications of the original electrometric method. These modifications included increasing the sample volume, increasing the reaction temperature, the use of different buffers and increasing or decreasing the incubation time ([9,10,11,14,17,19]).

The present electrometric method described for measurement of blood ChE activities in man depended mainly on the modifications introduced by Mohammad et al. ([19]). The method has been applied successfully for the determination of blood or tissue ChE activities in several animal species (see the introduction). However, the method has not been validated for use in man. The present study is the first attempt to standardize and validate the present electrometric procedure in man. The short 20-minutes one step incubation time and the 0.2 ml sample volume appeared to be suitable for the assay conditions to produce enzyme activity without interference with the buffering capacity in the reaction mixture. This is in agreement with our earlier finding in animals ([19,24]). The one step short incubation time of the described method would be useful in increasing the efficiency of the procedure for multiple samples when compared to the incubation period of more than 60 minutes of the original Michel's method ([15]). The method also decreases substantially handling of the reaction mixture as found in other electrometric methods ([9,10,11,14]) and eliminates the problem of color interference found in the spectrophotometric methods ([8,9,10,11]) by using a glass electrode. Further, it is inexpensive, like the Michel's method, as it uses commonly available laboratory equipment and reagents.

With regards to precision of the assay, the described electrometric method produced acceptable low coefficients of variation in the plasma and erythrocytes. This result documents within-laboratory precision of the assay ([8,9,10]) and agrees with the reported precision of the method in animals ([19,23,24]). In spite of the expected limitations of between-laboratories comparisons ([8,9,10,11,12]), the described method needs such a comparison in the future studies. In an attempt to establish preliminary reference range values for plasma and erythrocyte ChE activities, this study presents for the first time normal blood ChE activity in man as determined by the described electrometric method.

Separation of plasma from erythrocytes requires a suitable anticoagulant. Some anticoagulants such as oxalate and citrate cannot be used for blood samples intended for ChE measurement because they bind to calcium and magnesium ions necessary for the enzyme activity ([8,9]). Both anticoagulants used in the present study apparently did not interfere with the ChE assay, and they were recommended for this type of assay ([8]).

Quinidine specifically inhibits pseudo ChE activity in the plasma ([10,11,12,13]), thus permitting the estimation of true ChE in the sample. In the present study, the estimated % of true ChE activity in the human plasma (21.4%) correlates with those reported by others ([10,11]). The overall ChE activities in the plasma and erythrocytes also correlated with normal values reported by the Michel's method ([9,15]).

To further validate the described electrometric method and explore its efficiency in measuring ChE inhibition caused by antiChE, a series of in vitro (human plasma and

erythrocytes) and in vivo (mice) experiments were performed. In vitro inhibition of plasma and erythrocyte ChE activities by dichlorvos and carbaryl is in agreement with the reported antiChE actions of these insecticides ([2,3,19,24]). These results suggested the sensitivity of the described method for detecting ChE inhibition caused by organophosphates and possibly carbamates. However, further ChE inhibition cannot be excluded from this in vitro system during the 20-minute incubation period. Keeping in mind that the original electrometric method cannot be recommended for detection of ChE inhibition caused by carbamates ([16,17,18]), further in vivo experiments became warranted using the described method to validate it for such a condition.

Dosing of mice with dichlorvos and carbaryl induced signs of toxicosis characteristic of cholinergic overstimulation. The described method detected considerable (24-47%) ChE inhibition in the plasma, erythrocytes and brain of mice dosed with the insecticides. Similarly, the described method with incubation times ranging between 20-40 minutes, was efficient in detecting ChE inhibition induced by organophosphates and carbamates in various animal species ([19,20,21,22,23,24,25,26]). Further, the method was applied on blood samples obtained from individuals with a history of exposure to insecticides, and it detected 38 and 26% ChE inhibition in the plasma and erythrocytes, respectively. However, marginal inhibition is almost impossible to assess unless pre-exposure ChE values have been determined in advance for each subject ([4,5,7,8,9,10,11,12]). ChE inhibition should be at least 15% to be considered significant and 20-30% ChE inhibition is considered an indication of exposure ([2,7,8,9,10,11,12]). In the present study, 38 and 26% decreases in plasma and erythrocyte ChE activities, respectively, in subjects in contact with insecticides confirmed the exposure, although the subjects had no apparent illnesses.

In conclusion, the described electrometric method was simple, precise and efficient for rapid determination of ChE activity in the plasma and erythrocytes of man and it could be an additional economic and useful technique for monitoring exposure to ChE inhibitors.

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